REMARKS

Claims 12-22 are currently pending in the above-referenced application. However, in order to advance prosecution, Applicant has canceled claims 12-22 and has added new claims 23-30. As will be detailed below, claims 23-30 are supported by the specification.

1. IDS

The Examiner requests information regarding related cases to this case. 08/496,806 is currently pending, 08/543,117 has been abandoned and file wrappered to 08/932,517 and 09/005,875 is pending. The Examiner requests information of how each of these cases relates to the present application. In response, Applicant notes that application serial no. 09/005,875 issued as U.S. Patent No. 6,174,693 and is related to an immunological method to determine protein activity wherein a variety of proteins may be assayed with a single antibody on standard equipment; application serial no. 08/932,517 issued as U.S. Patent No. 6,172,750 and is directed to a method or system that utilizes fluorescence in a sample that would otherwise yield inaccurate results due to spectrally interfering components and application serial no. 08/496,806 is still pending and is directed to a method for increasing the range and sensitivity of a photometric assay. The two issued patents and the pending application are herewith submitted in an Information Disclosure Statement. Applicant has submitted these documents to comply with his duty of disclosure.

2. Written Description Rejection

Claims 12-22 are rejected under 35 U.S.C. §112, first paragraph, as containing subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor(s), at the time the application was filed, had possession of the claimed invention. It is asserted that many of

the limitations in the newly added claims appear to be new matter. For example, in claim 21 "and not cholesteryl esterase" is not found in the specification as originally filed. Applicant is requested to point out where in the specification as originally filed enablement is found for each feature in the claims.

In response, Applicants first note that claims 12-22 have been cancelled. New claims 23-30 have been added. New claims 23-30 are directed to a method for measuring activity of a protein that transports substances among donor/acceptor substances comprising:

(a) obtaining a sample comprising said protein;

(b) incubating said sample with (i) a donor substance labeled with a light emitter wherein light emitted from said light emitter increases with increasing activity of said protein and (ii) an acceptor dependent concentration (claim 23) or a protein dependent concentration (claim 27) light emission intensity quencher, wherein quenching of said light emission intensity increases with concentration of acceptor present in said sample and wherein said quencher is a normalization factor and

(c) detecting light emission intensity to determine activity of said protein.

As shown in the table below, there is support in the specification for each of the claims:

Claim	**Claim Phrase**	**Support in Specification**
23 and 27	a donor substance labeled with a light emitter wherein light emitted from said light emitter increases with increasing activity of said protein	p. 9, lines 19-23: "A suitable volume of the patient's plasma is incubated with the CE donor in buffer according the invention. The CE donor is comprised of a fluorescently labeled CE.....The fluorescence increases, in the case of NBC, over time as the plasma CETP transfers the fluorescent CE from the donor to endogenous lipoprotein particles".
23	an acceptor dependent concentration light emission intensity quencher, wherein quenching of said light emission	Page 7-descriptions of Figures 1 and 2 and page 10, last paragraph: "The present invention accounts for variable lipoprotein profiles by normalizing with a color

	intensity increases with concentration of acceptor present in said sample and wherein said quencher is a normalization factor	development reaction in response to cholesterol and... The development of color creates a quenching effect upon the fluorescence of the CE. Therefore, the greater the concentration of CE/TG/PL/protein, the greater is the color quenching effect upon the fluorescent label. This normalizes the fluorescent intensity for LDL concentration..."
24 and 28	the donor particle comprises a fluorescent lipid.	Page 4, lines 5-8: The present invention includes a CE donor with a fluorescent label on the CE. The fluorescent cholesteryl ester is 22-(N-7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino-23,24-bisnor-5-cholen-3-yl linoleate (NBD-CE). Page 9, lines 20-21: "The CE donor is comprised of a fluorescently labeled CE.
25	the light emission intensity quencher is ..... a colorimetric assay specific for lipids	Page 12, lines 13-16: "Increased color decreases the measurable fluorescence intensity of the activity due to color quenching of the fluorescent label thereby normalizing the results according to the endogenous lipoproteins present in the plasma. Page 14, lines 2-4: The normalization may be based on colorimetric techniques utilizing TG and or CE due to the presence of endogenous lipoproteins in the sample.
25 and 29	In which the light emission intensity quencher is selected from the group consisting of..... a chemiluminescent activity assay	Page 6, last paragraph: The activity parameter is established with a light emitting measurement technique that includes fluorescent ..... assays where the protein activity is assessed by a fluorimeter ..... was a change in light emission intensity. Last line, page 11: "For example, in the case of CETP, neutral lipids (CE and/or TG) may be used as the normalizing factor."
26 and 30	in which the donor is a cholesteryl ester having a fluorescent label wherein said	The assay changes color in response to the concentration of C/CE in the plasma sample [not responding to the fluorescent

	label blocks cholesteryl esterase activity and does not block cholesteryl ester transfer protein activity.	CE in the donor due to the fluorescent label blocking CEH or binding but not CETP].
27	A protein concentration dependent light emission intensity quenching component of said assay, wherein quenching of said light emission intensity increases with increasing said protein concentration, wherein said quencher acts as a normalization factor	Paragraph bridging page 13 and 14: "The normalization factor is applied according to the invention and may include normalization with respect to the lipid transfer protein by utilizing an immunoprecipitation technique with a CETP antibody."
28	the light emission intensity quencher is ..... a turbidimetric assay specific for protein	As above, paragraph bridging page 13 and 14

It is clearly evident that there is adequate written description for the new claims in the specification. Therefore, Applicant respectfully requests that the rejection be withdrawn.

3. The Rejection Under 35 U.S.C. §102(e)

Claims 12-22 are rejected under 35 U.S.C. §102(e) as being anticipated by Brocia. It is asserted that this rejection under 35 U.S.C. §102(e) might be overcome either by a showing under 37 CFR §1.132 that any invention disclosed but not claimed in the reference was derived from the inventor of this application and is thus not the invention "by another," or by an appropriate showing under 37 C.F.R §1.131.

Applicant, in response, notes that claims 12-24 have been canceled. Applicant has canceled claims 12-24 in order to advance prosecution. However, the cancellation of these claims should not be considered to be acquiescence to the Examiner's position. Applicant reserves the right to file subsequent continuation and/or divisional applications containing claims directed to the canceled subject matter.

Applicant asserts that new claims 23-30 are not anticipated by Brocia. Specifically, new claims 23-29 are directed to a method for measuring activity of a protein that transports substances among donor/acceptor substances comprising:

(a) obtaining a sample comprising said protein;

(b) incubating said sample with (i) a donor substance labeled with a light emitter wherein light emitted from said light emitter increases with increasing activity of said protein and (ii) an acceptor dependent concentration (claim 23) or a protein dependent concentration (claim 27) light emission intensity quencher, wherein quenching of said light emission intensity increases with concentration of acceptor or protein present in said sample and wherein said quencher is a normalization factor and

(c) detecting light emission intensity to determine activity of said protein.

Thus, the method of the present invention employs an internal standard, the quencher, which acts as a normalization factor. In contrast, the method disclosed in U.S. Patent No. 5,770,355 uses a lipid transfer protein assay which requires the addition of excess exogenous acceptor to determine lipid protein activity (see column 5, lines 13-17 and column 18, line 38-47). The sample's lipid transfer protein is only determined by the addition of the exogenous acceptor. Lipid protein transfer assays known in the art at the time of the filing of U.S. Patent No. 5,770,355 involved the use of both a donor and acceptor substance. One such example given was the Diagnescent assay. Applicant submits herewith as Appendix A product sheets for assays that are analogous to the Diagnescent assay. Given that the method of the present invention contains a quencher which acts as a normalization factor, it would follow that exogneous acceptor is not added.

However, even assuming *arguendo* that new claims 23-30 are anticipated by Brocia, Applicant notes that the subject matter disclosed and claimed in the instant application was not invented by "another". Robert Brocia is the sole inventor of the subject matter disclosed and claimed in both the instant application and in U.S. Patent No. 5,770,355. In response to the Examiner's request, a Declaration Under 37 C.F.R.

§1.132 is herewith submitted where applicant declares that he is the sole inventor of the subject matter disclosed and claimed in both the instant application and in U.S. Patent No. 5,770,355.

Therefore, Applicant asserts that in view of new claims 23-30 and the above arguments, the rejection under 35 U.S.C. §102(e) has been overcome. Therefore, Applicant respectfully requests that the rejection be withdrawn.

4. The Rejection Under 35 U.S.C. §101

Claims 12-22 are rejected under 35 U.S.C. §101 as claiming the same invention as that of claims 1-36 of prior U.S. Patent No. 5,770,355. This is a double patenting rejection. In the Examiner's view, the present claims, as written, encompass the same inventions as claimed in '355.

Applicants respectfully traverse the rejection. As noted above, claims 12-22 have been canceled. New claims 23-30 have been added. These new claims do not encompass the same inventions claimed in '355. The MPEP §804 II.A. states

> In determining whether a statutory basis for a double patenting rejection exists, the question to be asked is: Is the same invention being claimed twice? 35 U.S.C. 101 prevents two patents from issuing on the same invention. "Same invention" means identical subject matter....
>
> A reliable test for double patenting under 35 U.S.C. 101 is whether a claim in the application could be literally infringed without literally infringing a corresponding claim in the patent (citations omitted). Is there an embodiment of the invention that falls within the scope of one claim, but not the other? If there is such an embodiment, then identical subject matter is not defined by both claims and statutory double patenting would not exist.

It is clearly evident that the same invention is not being claimed in the '355 patent and in the instant application for a number of reasons. First, the method of the present invention is directed to a method for measuring activity of a protein that transports

substances among donor/acceptor substances without addition of exogenous acceptor. In contrast, for the most part, the claims of the '355 patent are directed to measuring lipid transfer protein activity with exogenous acceptor (in particular, see claim 23 of the '355 patent). Additionally, in particular, independent claims 1, 9, 11, 16, and 23 of the '355 patent specifically recite that fluorescent emission is used. In new claims 23-30 of the pending application, **light emission intensity** is detected (see step c). Claim 15 of the '355 patent is directed to using a CETP assay. New claims 23-30 of the pending application clearly cover methods for measuring the activities of other transfer proteins in addition to CETP. Claims 32 and 33 of the '355 patent are not even method claims. Claim 34 of the '355 patent is directed to a method for affecting lipid transfer protein activity; this is rather different from a method for measuring activity of a protein that transports substances among donor/acceptor substances, the method recited in new claims 23-30 of the pending application. Finally, as noted above, in the method of the present invention, the quencher acts as a normalization factor.

In view of new claims 23-30 and the above arguments, the double patenting rejection has been overcome. Therefore, Applicant respectfully requests that the rejection be withdrawn.

5. The Enablement Rejections

Claims 12-22 are rejected under 35 U.S.C. §112, first paragraph, as containing subject matter which was not described in the specification in such a way as to enable one skilled in the art to which it pertains, or with which it is most nearly connected, to make and/or use the invention. It is asserted that the application as originally filed does not enable the newly submitted claims that appear to be directed to an assay with some fluorescent component. It would appear there is a chemical reaction cascade involved in the invention but none is shown. The acronyms, in the Examiner's view, are not clear and are inconsistently applied. The Examiner points out the following specific instances: page 9 last full paragraph, such a method is not understood in context. On page 10

second paragraph last line, it is not seen how CETP mass is measured. On page 11 last paragraph, "the normalization factor includes a colorizing factor that reacts in response to the normalizing factor of choice." The example on page 12 is insufficiently detailed to follow. It is noted that neither data of any sort nor results of any determination are found in the specification. As written, one of skill in this art could not perform the claimed invention by following the teachings of the specification.

Again, as noted above, Applicant, in response, notes that claims 12-22 have been canceled. New claims 23-30 have been added. The specification, in Applicant's view, does enable new claims 23-30. As shown in the table above, there is certainly support for the new claims. The pending claims do not claim acronyms. With respect to page 10, second paragraph, Applicant notes that this statement was put in to show the advantages of having a normalization factor. It is noted that when no exogenous acceptor or normalization factor is added, CETP activity may appear to be higher even though there is no change in the amount (mass) of CETP present. With respect to page 11, last paragraph, a sufficient description of the normalization factor is provided on page 12. Finally, Applicant asserts that one of ordinary skill in the art could perform the claimed invention following the teachings of the specification. The fact that experimentation may be involved in making this determination, even if it may be time-consuming and laborious, it is not fatal, provided that the experimentation is routine, and does not require the exercise of inventive skill. *In re Wands,* 8 USPQ 2d (Fed. Cir. 1988); *Fields v. Conover*, 170 USPQ 276 (CCPA 1971). A sufficient description of an embodiment of the present invention is provided in the paragraph bridging pages 13 and 14 in the instant application.

Claims 12-22 are rejected under 35 U.S.C. 112, first paragraph, because the specification does not reasonably provide enablement for the following terms. The specification does not enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the invention commensurate in scope with these claims. Specifically, it is asserted that in claim 12 and all occurrences, the terms "an activity dependent light emission intensity component," and "a substrate

concentration dependent light emission intensity quenching component" lack enablement as it would require one of ordinary skill in this art undue experimentation to determine which such component would work in the instant invention.

In response, Applicant notes that claims 12-22 have been canceled. New claims 23-30 have been added. The terms "an activity dependent light emission intensity component," and "a substrate concentration dependent light emission intensity quenching component" are not recited in the new claims. However, new claims 23 and 27 recite (a) a donor substance labeled with a light emitter wherein light emitted from said light emitter increases with increasing activity of said protein and (b) an acceptor dependent concentration (claim 23) or a protein dependent concentration (claim 27) light emission intensity quencher, wherein quenching of said light emission intensity increases with concentration of acceptor present in said sample and wherein said quencher is a normalization factor. It would certainly not require one of ordinary skill in the art undue experimentation to determine whether these components would work in the instant invention. As noted above, a sufficient description is provided in the specification regarding the use of the donor substance and quencher (see, for example, pages 11-14 which describes the use of these components in measuring the activity of CETP present).

In view of new claims 23-30 and the above arguments, Applicant asserts that the pending claims are enabled by the specification. Therefore, Applicant respectfully requests that the rejection be withdrawn.

6. The Rejection Under 35 U.S.C. §112, Second Paragraph

Claims 12-22 are rejected under 35 U.S.C. § 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention. The rewritten claims are not understood and do not appear to be directed to the specification as originally filed. For example, "said light emission intensity represents said measurement of said enzyme activity" is not understood. In claim 21 "CETP" all acronyms should be spelled out in the first

occurrence in the claims. In general, the claims lack antecedent basis and have inconsistent tenses to they cannot be properly interpreted.

In response, as noted above, claims 12-22 have been canceled. New claims 23-30 have been added. These new claims in Applicant's view resolves any 35 U.S.C. §112, second paragraph issues. Therefore, Applicant respectfully requests that the rejection be withdrawn.

7. Miscellaneous

The title of the invention is not aptly descriptive. A new title is required that is clearly indicative of the invention to which the claims are directed. In response, a new title is provided.

The disclosure is objected to because of the following informalities: The headings in the specification are nonstandard, for example, no Brief Description of the Drawings is found. There are sufficient typographical errors in the specification, including inexplicable brackets, to affect the meaning of the writing. Note no amendment can include brackets to amend the specification that contains brackets. Appropriate correction is required. In response, Applicant herewith submits a redlined copy of the original application as well as a supplemental application containing the appropriate corrections. The Supplemental Application should overcome the objections to the disclosure.

The new title and Supplemental Application have addressed the concerns raised in the Office Action. Therefore, Applicant respectfully requests that the objections be withdrawn.

8. Conclusion

In view of the above, it is respectfully submitted that all claims are in condition for allowance. Early action to that end is respectfully requested. The Examiner is hereby

invited to contact the undersigned by telephone if there are any questions concerning this amendment or application.

Respectfully submitted,

Date: 4/17/01

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~~Patent Application of Robert W. Brocia~~

~~For~~

A METHOD TO DETERMINE THE ACTIVITY OF ~~AN ENZYME~~A PROTEIN THAT TRANSPORTS SUBSTANCES BETWEEN DONOR/ACCEPTOR SUBSTANCES

~~Background~~ Field of the Invention

This invention relates generally to the field of biochemistry and clinical chemistry. More particularly, the invention is a novel method to measure the activity of a~~n enzyme~~an enzyme.

~~Background - Description of Prior Art~~Background of the Invention

Conventional characterization of a protein with enzymatic activity is expressed in terms of product formed per mass of protein present per unit time. This is referred to as the specific activity of the protein. For example, a protein that hydrolyzes a substrate to form products X and Y may be described as having a specific activity of moles of X or Y formed per gram of protein per minute

This specific activity determination requires two separate laboratory procedures 1) a measurement of the amount of product formed (moles) in a certain amount of time by a sample of the protein, and 2) an assay to determine the protein mass (gms) present in the sample. Normally the protein mass is measured in a colorimetric assay such as a ~~lowry~~ Lowry protein assay or a BCA assay. The mass determination requires that a

portion of the protein be sacrificed so that it may be reacted with the ~~lowry~~ Lowry reagents.

The conventional specific activity characterization is limited to proteins that have bond breaking and/or making activity such as lipases, esterases, oxidases and others. The bond breaking and/or making activity is defined herein for ease of explanation of the invention as activity that makes and/or breaks chemical bonds.

~~The present invention is useful for characterization of these proteins and other proteins that do not make and/or break bonds. For example, proteins such as cholesteryl ester transfer protein (CETP), microsomal [triglyceride] transfer protein (MTP), phospholipid transfer protein (PLTP) do not cleave a chemical bond. These proteins express their activity by transporting lipids among donor/acceptor sites, it is believed that the activity of these proteins is modulated according to the liquid crystalline states of their respective substrates and may follow an entropy gradient and in addition there are proteins that do make/break bonds but are unusual in that there activity changes according to the physical state of the substrate, i.e. lipase. All these lipid active proteins CETP,MTP, PLTP and lipase and others including lecithin cholesterol acyl transferase (LCAT), ACAT and others including enzymes that react with protein substrates of varying liquid crystalline states may be characterized by the present invention. The present invention is also useful to characterize enzymes that are described by convention means.~~

~~The present invention is utilized to characterize all enzymes and normalizes the activity of the enzyme by the amount of substrate or protein mass present. The invention accomplishes this for use such as in the clinic where samples of physiological fluids may contain protein activity. The samples may demonstrate varying protein activity from patient to patient. The differences in activity solely due to the amount of protein present in the sample of active mass must be discerned from activity due to varying amounts of substrate present in the sample.~~

For example, cholesterol ester transfer protein (CETP) is a plasma protein that shuttles lipids among lipoproteins (~~including~~which includes high density lipoprotein [~~(~~HDL~~],~~) low density lipoprotein [~~(~~LDL~~]~~) intermediate density lipoprotein [~~IDL~~](IDL), very low density lipoprotein [~~(~~VLDL~~]~~) and chylomicrons. If the plasma CETP activity is measured in a group of patients, the measurement must take into account the concentration of lipoprotein particles present in each sample. This is conventionally achieved by adding an excess of VLDL or LDL to the test sample to normalize the acceptor concentration among each sample. The conventional CETP assay would include a volume of a ~~patients~~patient's plasma combined with a CETP compatible cholesteryl ester (CE) donor particle. The CE is labeled so that the CE mass may be quantitated after the protein shuttles the CE from donor to acceptor. A suitable acceptor is added to the plasma and donor mixture in a buffer to replicate physiological conditions. The donor, acceptor, plasma and buffer mixture is incubated. After incubation, the assay is analyzed to determine the amount of labeled CE transferred from donor to acceptor.

The added acceptor is in excess and compensates for the differences in lipoprotein particle numbers associated with each patient's plasma lipoprotein profile. If the acceptor were not added, the endogenous lipoproteins would accept the transferred CE and results of the test would vary based not only upon activity of the protein but also according to teach patient's lipoprotein profile.

~~The present invention improves upon the conventional methods of determining CETP activity in a plasma sample. The present invention eliminates the acceptor of the previous method. Thus reducing variability associated with VLDL preparations and further eliminates hazards involved with the handling of human blood products. The components of the method are more stable and have a longer shelf life without VLDL.~~

~~The present invention includes a CE donor with a fluorescent label on the CE. The fluorescent cholesteryl ester is 22-(N-7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino-23,24-bisnor-5-cholen-3-yl linoleate (NBD-CE). The CE with fluorescent label (NBD) included in the donor is formatted so as CETP activity causes a change in fluorescence over time. Furthermore, the invention includes the fluorescent label to block reaction with non-CETP proteins such as cholesteryl esterase.~~

Objects and Advantages

Accordingly, several objects and advantages of ~~my~~ the invention are ~~An~~ an enzyme assay that internally controls the assay result for factors that affect enzyme activity.

———It is also a ~~principle~~ principal object of the invention to characterize a protein activity in one assay.

———It is another object of the invention to characterize a protein activity in a clinical sample for the purpose of diagnosis of a disease.

Still further objects and advantages will become apparent from a consideration of the ensu~~r~~ing description and accompanying drawings.

Summary of the Invention

The present invention is a method to measure protein ~~that~~ wherein said method internally controls for factors that affects protein activity such as substrate concentration.

The present invention is useful for characterization of these proteins and other proteins that do not make and/or break bonds. For example, proteins such as cholesteryl ester transfer protein (CETP), microsomal [triglyceride] transfer protein (MTP), phospholipid transfer protein (PLTP) do not cleave a chemical bond. These proteins express their activity by transporting lipids among donor/acceptor sites, it is believed that the activity of these proteins is modulated according to the liquid crystalline states of their respective substrates and may follow an entropy gradient and in addition there are proteins that do make/break bonds but are unusual in that there activity changes according to the physical state of the substrate, i.e. lipase. All these lipid active proteins CETP, MTP, PLTP and lipase and others including lecithin cholesterol acyl transferase (LCAT), ACAT and others including enzymes that react with protein substrates of varying liquid crystalline states may be characterized by

the present invention. The present invention is also useful to characterize enzymes that are described by conventional means.

The present invention is utilized to characterize all enzymes and normalizes the activity of the enzyme by the amount of substrate or protein mass present. The invention accomplishes this for use such as in the clinic where samples of physiological fluids may contain protein activity. The samples may demonstrate varying protein activity from patient to patient. The differences in activity solely due to the amount of protein present in the sample of active mass must be discerned from activity due to varying amounts of substrate present in the sample.

The present invention improves upon the conventional methods of determining CETP activity in a plasma sample. The present invention eliminates the acceptor of the previous method. Thus reducing variability associated with VLDL preparations and further eliminates hazards involved with the handling of human blood products. The components of the method are more stable and have a longer shelf life without VLDL.

The present invention includes a CE donor with a fluorescent label on the CE. The fluorescent cholesteryl ester is 22-(N-7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino-23,24-bisnor-5-cholen-3-yl linoleate (NBD-CE). The CE with fluorescent label (NBD) included in the donor is formatted so as CETP activity causes a change in fluorescence over time. Furthermore, the invention includes the fluorescent label to block reaction with non-CETP proteins such as cholesteryl esterase.

Brief Description of the Drawings

Fig. 1 is an illustration that depicts the light emitting enzyme activity determination step of the assay followed by the normalization step of the assay in three different samples.

Fig. 2 shows the invention as immunoturbidometric normalization is applied.

Fig. 3 shows the invention applied to lipid transfer protein, specifically CETP.

~~**Summary**~~

~~The present invention is a method to measure protein that internally controls for factors that affects protein activity such as substrate concentration.~~

~~**Preferred Embodiment – Description**~~**Detailed Description of the Invention**

The present invention is a method to characterize protein activity wherein the characterization is not calculated from separately determined values such as activity and protein mass. The present invention involves an enzyme activity parameter and a normalization parameter. The activity parameter method according to the invention is a protein activity assay where in the result, i.e., net chemiluminescence or fluorescence of the activity assay will change when an assay to determine the concentration

of the protein, substrates or other factor of interest in performed if the change in the result is not due to a change in protein activity.

The present invention is a clinical diagnostic test wherein the result of the test on a sample solution is obtained in one determination from one instrument yet the result that is detected by the instrument is due to a combination of more than one independent spectrophotometric assay. One spectrophotometric assay emits light and another alters the optical density of the assay. The combined effect is measured as one result by one instrument.

A clinical diagnostic test wherein the result is measured by one instrument in one determination and the result is a function or more than one chemical indicator where each chemical indicator functions independently.

The invention is a screen for inhibitors or promoters of protein activity for the purpose of pharmaceutical drug development.

This invention disclosure provides several examples of different fluorescent activity assays where the emitted light is varied according to protein activity and then adjusted with respect to the substrate concentration or, alternatively, adjusted with respect to protein concentration by immunological techniques or ~~colonmetric~~ colorimetric assay.

The activity parameter is established with a light emitting measurement technique that includes fluorescent and chemiluminescence enzyme activity assays where the protein activity is assessed by a fluorimeter or illuminometer was a change in light emission intensity.

~~Preferred Embodiment -- Operation~~

Fig. 1 illustrates the enzyme activity dependent fluorescence or chemiluminescence portion of the method according to the invention followed by the substrate or product normalization portion of the invention. In Fig. 1, enzyme and substrate interactions **10**, **11**, **12** are depicted so substrate concentration is increasing from **10** to **12**. After enzyme substrate interaction fluorescent or chemiluminescent products **20, 21, 22** are obtained. The products are of varying fluorescent or luminescent intensity according to varying substrate concentration. Normalization factor **30, 31, 32** is interacted with the light emitting mixture and color develops **40, 41, 42** according to the increasing substrate concentration present. The developed color causes an increasing optical density thus modulating the light emission intensity from the mixture. The fluorimeter or illuminometer or other light detecting instrument would collect the substrate dependent light emission intensity.

FIG. 2 illustrates another embodiment of the invention. Enzyme and substrate mixture **100** yields an activity dependent light emitting product

110. Normalization factor **120** is an antibody specific for the enzyme and immunoprecipitation components. Resulting mixture **130** is turbid ~~by y~~ precipitated ~~antibodylenzyme~~ antibody/enzyme complex and the turbidity blocks the emitted light from the light emitting product formed by the enzyme activity. The results are light emission intensity that is normalized by enzyme mass present.

EXAMPLES

The following are specific examples involving certain proteins of interest to provide a complete understanding of the invention:

———In the diagnosis of heart disease the lipid transfer proteins seem to play an important role. The many complex interactions involving lipids make a clear path for intervention difficult to perceive. For example, the relationship between saturated ~~fa~~cts in a patient's diet and the patient's total plasma cholesterol is believed to be a factor of solubility. If an amount of saturated fat is packaged in a lipoprotein particle core, the lipoprotein particle core will also solubilize cholesteryl ester. Cholesteryl ester has an increasingly limited solubility in triglycerides (TG) of increasing saturation which means a fixed mass of cholesteryl ester will require more lipoprotein particles for solubilization if the triglyceride is of a more saturated type. Fewer lipoprotein particles are required if the TG is of an unsaturated type.

Therefore, a saturated fat diet will generate more ~~lipoportion~~ lipoprotein particles to move about an equivalent amount of cholesteryl ester. Additionally, free cholesterol will partition into the lipoprotein fraction

at about 3% solubility factor from the plasma red cells boosting plasma cholesterol in a saturated fat diet dependent manner.

Cholesteryl ester transfer proteins (CETP) is a protein present in normal human plasma. CETP transfers lipids among lipoprotein particles. Of the most important of these transfer events is the transfer of cholesteryl esters (CE) from high density lipoprotein (HDL) to low density lipoprotein (LDL) or very low density lipoprotein (VLDL).

This example is important to express the invention in terms of an unusual enzyme such as CETP because ~~with~~ with CETP a product is not ~~formed by~~formed by breaking chemical bonds as with other enzymes. Proteins such as cholesteryl ester transfer protein (CETP), microsomal [triglyceride] transfer protein (MTP), phospholipid transfer protein (PLTP) do not cleave a chemical bond. These proteins express their activity by transporting lipids among donor/acceptor sites, it is believed that the activity is according to an entropy gradient and in addition there are proteins that do make/break bonds but are unusual in that their activity also changes according to the physical state of the substrate, i.e., lipase. These lipid active proteins, CETP, ~~MTP~~, MTP, PLTP and lipase and others including lecithin cholesterol acyl transferase (LCAT), acyl cholesterol acyl transferase (ACAT) and other enzymes not lipid active, including enzymes that react with protein substrates according to the liquid crystalline states of the protein substrates.

Conventionally, CETP activity cannot be expressed as a specific activity because there is no product formed by making/breaking chemical bonds. The following example presents one embodiment of the present invention as a clinical method to measure CETP activity:

———A suitable volume of the patient's plasma is incubated with the CE donor in buffer according to the invention. The CE donor is comprised of a fluorescently labeled CE. The fluorescent label ~~included~~ includes NBD. The fluorescence increases, in the case of NBD, over time as the plasma CETP transfers the fluorescent CE from the donor to endogenous lipoprotein particles.

The fluorescence intensities among the group of samples will be varied according to the CETP activity in the samples *and* any variability among the concentrations of lipoproteins in samples. For example~~s~~, differences among a patient's LDL cholesterol will be reflected in the activity of CETP. This is explained by the LDL cholesterol values resulting from the actual number of LDL particles present in the plasma. So a patient's plasma sample that is high in LDL cholesterol has more LDL particles in suspension then a patient who has a low LDL cholesterol. The patient's plasma with high LDL cholesterol will appear to have a high CETP activity when in fact the apparently high activity is due to the greater number of LDL particles available to accept transfer of the NBD-CE or other fluorescent ~~cholesterol~~cholesteryl ester.

Furthermore, given two patients with identical CETP activity and lipoprotein profiles, one has a meal and the other is fasted, the CETP is

measured with donor and no exogenous acceptor. The fed patient will have an apparent increase in CETP activity because of chylomicron particles that circulate in the plasma after meals as a normal component of digestion. The chylomicrons will behave as acceptor of CE. The CETP activity will appear to be higher in the fed patient when in fact CETP mass may not change.

The present invention accounts for variable lipoprotein profiles by normalizing with a color development reaction in response to cholesterol and/or triglyceride and/or phospholipid and/or protein. The development of color creates a quenching effect upon the fluorescence of the CE. Therefore, the greater the concentration of CE/TG/PL/protein the greater is the color quenching effect upon the fluorescent label. This normalizes the fluorescent intensity for LDL concentration.

The present invention provides an assay that yields one value representing the activity of an enzyme. The invention accounts for enzyme specific variables that may normally affect the activity of a protein in an activity assay.

The invention is applied to the measurement of activity of CETP present in a patient's plasma through the use of a synthetic donor particle. The donor particle provides a source of fluorescently labeled CE to the protein. The CE is present in a self-quenched state in the core of the donor particle. Therefore, when the CE is removed from the core by the protein a measurable increase in fluorescent occurs. The CETP shuttles the

cholesteryl ester from the donor particle to endogenous lipoproteins present in the plasma sample. These endogenous lipoproteins act as acceptor particles. The more active the CETP the higher is the fluorescence after a period of time.

The patient's plasma will have a variable amount of endogenous lipoprotein acceptor particles dependent upon the patient's particular plasma lipoprotein profile. Therefore, the fluorescence increased from the activity of the CETP present in a patient's plasma is dependent on the concentration of lipoproteins present in their plasma as well as the activity of the CETP. The invention provides a normalization factor based upon substrates of the CETP which include endogenous cholesteryl esters and triacyl glycerols, the major core components of endogenous HDL, LDL and VLDL. The normalization factor may also include phospholipids or cholesterol or any component present in the sample that would have an effect on CETP activity.

The normalization factor includes a colorizing factor that reacts in response to the normalizing factor of choice. For example, in the case of CETP, neutral lipids (CE and/or TG) may be used as the normalizing factor. A mixture of cholesteryl esterase (CEH) >100 U/L, cholesterol oxidase (CO) 300 U/L, peroxidase (PO) 1000 U/L, 4-aminoantipyrine 0.3 mmol/L, p-hydroxybenzenesulfonate 30 mmol/L in a buffer at pH 6.5 (in the case of CE as the normalization factor) is added to the incubation comprising fluorescent CE/donor and plasma. The CEH hydrolyzes any non-fluorescent cholesteryl esters to cholesterol. The cholesterol is oxidized by CO to cholest-4-en-3-one and hydrogen peroxide. The hydrogen peroxide is

coupled with 4-aminoantipyrine and p-hydroxybenzenesulfonate in the presence of PO to yield a quinoneimine dye. The assay changes color in response to the concentration of C/CE in the plasma sample [not responding to the fluorescent CE in the donor due to the fluorescent label blocking CEH ~~or~~ binding but not CETP]. The higher the concentration of the C/CE from the endogenous plasma lipoproteins, the darker the color from the colorimetric assay. Increased color decreases the measurable fluorescence intensity of the activity assay due to color quenching of the fluorescent label thereby normalizing the results according to the endogenous lipoproteins present in the plasma.

Normalization for TG in the assay includes the addition of a mixture of reagents that respond to TG with a color development. For example, a mixture of reagents that include: adenosine triphosphate (ATP) 0.3 mmol/L, magnesium salt 3 mmol/L, 4-aminoantipyrine 0.15 mmol/L, sodium N-ethyl-N-(3-sulfopropyl)-m-anisidine 1.69 mmol/L, lipase 50,000 U/L (LP), glycerol kinase 1000 U/L (GK), glycerol phosphate oxidase 2000 U/L (GPO), peroxidase (PO) and buffer at pH 7.0 is added to the assay. Triglycerides are hydrolyzed by LP to glycerol and free fatty acids. Glycerol is phosphorylated by ATP forming glycerol-1-phosphate (G-1-P) and adenosine-5-diphosphate in a reaction catalyzed by GK. G-1-P is then oxidized by GPO to dihydroxyacetone phosphate and hydrogen peroxide. A quinoneimine dye is produced by the PO catalyzed coupling of 4-aminoantipyrijne and sodium N-ethyl-N-(3-sulfopropyl)-m-anisidine with hydrogen peroxide. The color develops according to the concentration

of triglyceride present in the assay. The color affects the fluorescence intensity reading determined by the instrument.

The invention ~~that~~ internally normalizes CETP activity based upon CE or TG concentration of a patient's plasma.

It is important to note that although the normalization components of the invention are indicator enzymes which hydrolyze specific substrates present in plasma to effect a optical density change, the indicator enzymes do not react with the fluorescently labeled cholesteryl ester of the donor particle.

The present invention is applied to lipid transfer proteins, such as CETP according to the figures as follows: in Fig. 3, a sample **300**, is a plasma or other physiological sample that may include variable concentrations of lipoproteins and CETP. A volume of the sample is incubated in an appropriate buffered incubation mixture **310** with fluorescent cholesterol ester donor particle **320** comprised of self-quenched fluorescently labeled cholesterol ester **330**. During incubation CETP **340** present in the sample transfers fluorescently labeled cholesteryl ester to any acceptors **350**, such as lipoproteins, present in the sample. As transfer occurs there is an increase in fluorescence intensity in the incubated sample. The increase is dependent upon the activity of lipid transfer protein and the concentration of endogenous acceptor present in the sample. The normalization factor is applied according to the invention and may include normalization with respect to the lipid transfer protein by utilizing an immunoprecipitation technique with a CETP antibody. The normalization

may be based on colorimetric techniques utilizing TG and CE due to the presence of endogenous lipoproteins in the sample.

~~Conclusions, Ramifications, and Scope~~

Accordingly, it can be seen that the invention provides a convenient technique to characterize a protein activity in one instrument with the inclusion of at least two spectrophotometric assays.

Although the description above contains many specificities, these should not be construed as limiting the scope of the invention but as merely providing illustrating of some of the presently preferred embodiments of this invention. Various other embodiments and ramifications are possible within its scope. For example, virtually any protein activity may be assayed with one internally controlled assay according to the invention.

Thus the scope of the invention should be determined by the appended claims and their legal equivalents, rather than by the examples given.

~~**Claims: What is claimed is:**~~

~~1. A method to measure enzyme activity comprising;~~
~~a protein activity dependent light emitting component, and;~~
~~a substrate concentration dependent light quenching component.~~

~~2. The method of claim 1 wherein said protein activity dependent light emitting component includes a donor particle comprised of fluorescent lipid and said substrate concentration dependent light quenching component includes a colorimetric assay specific for neutral lipids.~~

~~3. The method of claim 1 wherein said protein activity dependent light emitting component includes a donor particle comprised of fluorescent lipid and said substrate concentration dependent light quenching component includes a turbidometric assay specific for a protein.~~

~~4. A cholesteryl ester comprised of a fluorescent label wherein said label blocks cholesteryl esterase activity and does not block cholesteryl ester transfer protein activity.~~

~~5. A method to determine enzyme activity comprising an activity parameter and a normalizing parameter.~~

~~6. The method of claim 5 wherein said activity parameter is a light emitting assay and said normalizing parameter quenches light emission.~~

7. The method of claim 6 wherein said normalization parameter includes turbidity from immunoprecipitation products.

8. A method to determine one clinical diagnostic value from a sample wherein said method comprises more than one spectrophotometric assay.

9. The method of claim 8 wherein said spectrophotometric assay includes an assay that increases the optical density of the sample solution.

10. A clinical diagnostic test wherein the result is measured by one instrument in one determination and said result is a function of more than one independently functioning chemical indicator.

11. A method to assay plasma CETP activity without exogenous acceptor.

A METHOD TO DETERMINE THE ACTIVITY OF AN ENZYME

Abstract:

A method to determine the activity of a protein wherein the method internally controls for substrate concentration. The method includes a fluorescent or chemiluminescent activity assay followed by a normalization step that quenches the light detected by an instrument measuring the assay. The quenching is in response to a concentration of substrate or protein present in the assay.

WHAT IS CLAIMED IS:

23. A method for measuring activity of a protein that transports substances among donor/acceptor substances comprising:

(a) obtaining a sample comprising said protein;

(b) incubating said sample with (i) a donor substance labeled with a light emitter wherein light emitted from said light emitter increases with increasing activity of said protein and (ii) an acceptor dependent concentration light emission intensity quencher, wherein quenching of said light emission intensity increases with concentration of acceptor present in said sample, wherein said quencher acts as a normalization factor and

(c) detecting light emission intensity to determine activity of said protein.

24. The method according to claim 23, in which the donor particle comprises a fluorescent lipid.

25. The method according to claim 23, in which the light emission intensity quencher is a colorimetric assay specific for lipids.

26. The method according to claim 23, in which the donor comprises a cholesteryl ester having a fluorescent label wherein said label blocks cholesteryl esterase activity and does not block cholesteryl ester transfer protein activity.

27. A method for measuring activity of a protein that transports substances among donor/acceptor substances comprising

(a) obtaining a sample comprising said protein

(b) incubating said sample with (i) a donor substance labeled with a light emitter wherein light emitted from said light emitter increases with increasing activity of said protein and (ii) a protein dependent concentration light emission intensity quencher, wherein quenching of said light emission intensity increases with concentration of protein present in said sample, wherein said quencher acts as a normalization factor and

(c) detecting light emission intensity to determine activity of said protein.

28. The method according to claim 27, in which the donor particle comprises a fluorescent lipid.

29. The method according to claim 27, in which the light emission intensity quencher is a turbidimetric assay specific for protein.

30. The method according to claim 27, in which the donor comprises a cholesteryl ester having a fluorescent label wherein said label blocks cholesteryl esterase activity and does not block cholesteryl ester transfer protein activity.

ABSTRACT

A method to measure protein wherein said method internally controls for factors that affects protein activity such as substrate concentration.

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